The Study of *Arabidopsis thaliana* Trichomes for Source of Chemical Defense Against Pathogens

Laura Bruner

Advised by Dr. Erich Grotewold

The Ohio State University

Department of Plant Pathology

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Abstract

Trichomes on plants, such as *Arabidopsis*, may provide a physical barrier to pathogen infection. Evidence for *Arabidopsis* trichomes to secrete chemicals or signals to inhibit pathogen attack is not presently available. The pathogenesis-related protein, PR-1 is expressed in several plant species after infection with *Pseudomonas syringae pv. phaseolicola* (*P. s. pv. phaseolicola*) or its *hrp* mutant. Mutations in *Pseudomonas* in the hypersensitive response and pathogenicity genes are called *hrp* mutants, which is a Type Three Secretion System deficient (TTSS-) strain. The main objective of this study is to observe the induction of PR-1 proteins in trichomes, Columbia wildtype leaves, and Columbia *gl1* (*GLABROUS 1*) mutant leaves when inoculated with *P. s. pv. phaseolicola*. The *hrp* mutant serves as the control with little or no induction of PR-1. Previous studies show *P. s. pv. phaseolicola* and the *hrp* mutant induce PR-1 proteins in *Arabidopsis* Columbia wildtype leaves. Testing of the objective will be accomplished by growing *Arabidopsis* Columbia wildtype and *gl1* mutant seedlings and inoculating with *P. s. pv. phaseolicola*, *hrp* mutant, or distilled water as control treatment. RNA from leaves and trichomes will be analyzed by performing real time Reverse-Transcription Polymerase Chain Reaction (RT-PCR). A technique was established to efficiently isolate trichomes from the surface of *Arabidopsis*'s leaves and study PR-1 in trichomes specifically. PR-1 is expressed in wildtype trichomes after induced by *P. s. pv. phaseolicola* independent of TTSS. *P. s. pv. phaseolicola* induced PR-1 in wildtype leaves at significantly high levels (one sided t-test, $p<0.05$). *Hrp* mutant (TTSS-) induced significantly high level of PR-1 in *gl1* mutant leaves (one sided t-test, $p<0.05$). Evidence of PR-1 in trichomes strongly suggests that trichomes have a defense mechanism to detect pathogen infection. This study

provides evidence for loss of the *GL1* genes to play a role in defense mechanisms against pathogens. Results from this study and future studies will be applicable to pathogen induced trichome defense mechanisms.

Introduction

Arabidopsis thaliana, a weed of the mustard lineage, is a model organism for study because of a number of reasons. It takes six to eight weeks to grow from seed to seed set; it is a small plant that allows for many plants and multiple replications to be grown in a small space. The entire genome is sequenced and comprises 30,000 genes. This organism is used for genetic studies because most of the gene functions are known and there is small amount of DNA per cell (Campbell and Reece 2005).

A trichome is a protuberance that is similar to a hair coming from the epidermis of an aerial tissue. Trichomes can vary based on cell count, density, and presence or absence of cytoplasm. There is a positive correlation in plants between trichome density and the resistance to pathogens and herbivores. Dicotyledonous angiosperms can contain glandular trichomes. Glandular trichomes can secrete toxins that poison or paralyze pests. The trichomes contain exudates to help protect the plant so when the insect's chemoreceptor comes in contact with the trichome it does not eat the host. This allows the host not to be damaged (Levin 1973).

The biochemical interactions that occur between pathogen and host create signals that the pathogen uses to distinguish between different hosts and whether or not to infect the host. Pathogens can infect hosts by direct penetration through natural openings, wounds, and across cell walls. Parts of the pathogen can act as elicitors for plant defense. Elicitors can determine whether or not a host is resistant to the pathogen. If the elicitor

induces the genes to be transcribed that are needed for the host defense then that makes the pathogen avirulent. If the elicitor is suppressed; it leads to growth and development of a disease. Pathogens such as bacteria and virus must first attach to the surface of the host. The pathogens adhere by mixtures of polysaccharides, glycoproteins, lipids, and fibrillar materials which are on the surface or tips of the pathogen. Virus pathogens and fastidious bacteria enter the cells directly by the penetration of their vector. Pathogens can secrete enzymes, toxins, growth regulators, and polysaccharides that can help in the development of the disease. The main interaction between a pathogen and its host is biochemical. The resistance of the host depends more on the chemicals produced before or after infection than the physical barriers (Agrios 2005).

There are two genetic resistance types in plant-pathogen interactions: quantitative and qualitative. Quantitative resistance is the effect of many minor genes and qualitative resistance is controlled by one gene with many impacts. The 'gene-for-gene' idea is that a host's resistance gene (R) corresponds to the pathogen's avirulence gene (Av). Resistance and avirulence are dominant to susceptibility and virulence, respectably with both R and Av needing to be present in the host for resistance to occur. 'Super-races' of pathogens can occur in agriculture where the pathogen can surmount the all the R genes. There is a correlation of broadly virulent pathogens being present in plant varieties that are very resistant, and avirulent pathogens being a part of susceptible varieties. Host plants can carry many resistance genes depending what pathogens are seen in the environment (Burdon and Thrall 2003).

A plant has an innate immune system that is designed to detect and produce a response to pathogen-derived molecules. One molecule of innate immune system is

Pathogen-Associated Molecular Patterns (PAMPs) or Microbe-Associated Molecular Patterns (MAMPs). Lipopolysaccharide (LPS) and/or flagella from bacteria or chitin and ergosterol from fungus can serve as an elicitor to trigger plant's PAMP receptor. The response to PAMPs is to produce pathogenicity related protein, PR in the plant. PAMPs are needed by the pathogen for survival and are therefore a successful way to detect "nonself" molecules. Another molecule is type three effector proteins that enhance the virulence of bacteria. The plant's R protein triggers a defense response to the type three effector proteins that ultimately leads to host cell death or hypersensitive response (HR). Induction of a response will not occur in the presence of a type three effector when the corresponding R protein is absent (Kim et. al. 2005).

P. s. pv. phaseolicola is a gram-negative bacteria that causes halo blight on beans. The bacteria use a TTSS to infiltrate the host cells with over 40 type three effector proteins (Kim et. al. 2005). *P. s. pv. phaseolicola* and its *hrp* mutant (TTSS-) is nonpathogenic on *Arabidopsis thaliana*, has PAMP Triggered Immunity (PTI), and does not cause a HR (personal communication from Dr. David Mackey). Prevention of HR can occur by inhibition defense signaling pathways or programmed cell death machinery (Ham et. al. 2007).

The morphology and function of trichomes can change with tissues and species. Morphology differs at the subcellular, cellular, and organ systems. Glandular trichomes produce exudates or mainly terpenoids oils that can be altered to bring about disease resistance, attraction of pollinating insects, and to synthesis compounds of metabolism that are not in the pathway. Trichomes arise from protodermal cells and the structures appear early in tissue differentiation. Manipulating exudates in some plants can help understand

the role it plays in pest interaction with the plant. Terpenoids can be harmful or beneficial to insects but the species, concentration, other chemicals, and the mode contact can influence the response to the exudates. Exudates can have a physiochemical property of being sticky and can release chemicals to help in the plant's defense (Wagner 1991).

There are a number of genes that control trichome development. *GLABROUS 1* (*GL1)* is important for the initiation and spacing of *Arabidopsis* trichomes. Loss of *GL1* results in a nonpubescent leaves. *TRANSPARENT TESTA GLABRA 1* (*TTG1*) gene, like *GL1*, plays a role in trichome initiation and spacing (Oppenheimer et. al. 1991). The frequency of trichome initiation and spacing is regulated by the gene *GLABROUS 2* (*GL2*) (Ohashi and Oka 2002).

Trichomes can secrete chemicals that are toxic to impede attack or infection. Some examples include *Nicotiana tabacum* (tobacco), *Helianthus annuus* (sunflowers), *Datura metel* (jimson weed), and *Mentha* (peppermint). A study on wild radishes with aphids was performed to see the effects of plant fitness when attacked by an herbivore. The experiment proceeded by introducing a caterpillar larva on a leaf in the induced plant treatment. In leaf damage control treatment, one leaf was cut off to be the same amount of damage that occurred in the induced plant. The third treatment was the overall control. The induced plants responded to the damage by increasing defensive mustard oil glycosides concentration and densities of trichomes. For the control treatments, there was a higher percentage of plant death and leaf damage control than in the induced plants (Agrawal 1998). Leaf water washed tobacco with T-phylloplanins inhibits disease caused by the pathogen *Peronospora tabacina* that causes blue mold disease by its spores. T-phylloplanins are proteins that are present in the short glandular trichomes. RNA

interference (RNAi) was used as a way to show that *T-phylloplanin* gene in tobacco stops the synthesis of T-phylloplanin mRNA and protein. RNAi works by short-interfering RNAs (siRNA) and microRNA (miRNA) binding to other RNA to increase or decrease its functionality. The RNAi plants to be unable to resist *P. tabacina* spores and leaf infection resulting in plants being subject to disease (Kroumova et al. 2007).

Problem Identification and Justification

Trichomes on plants, such as *Arabidopsis*, may provide a physical barrier to pathogen infection. Evidence for *Arabidopsis* trichomes to secrete chemicals or signals to inhibit pathogen attacks is not presently available. The pathogenesis-related protein, PR-1 is expressed in several plant species after infection with *Pseudomonas syringae pv. phaseolicola*. Mutations in *Pseudomonas* in the hypersensitive response and pathogenicity genes are called *hrp* mutants, which is a Type Three Secretion System deficient (TTSS-) strain. PR-1 proteins are expressed in leaves when infected with *P. s. pv. phaseolicola* or its *hrp* mutant (fig. 1A, Ham et. al. 2007). Trichomes of Columbia wildtype may produce and/or accumulate PR-1 proteins after induced with *P. s. pv. phaseolicola* or its *hrp* mutant. The *gl1* mutant in the Columbia ecotype is deficient in trichomes and can be used as a control for trichome dependent defense responses. The results may be used to do further studies on the specific pathogenic proteins produced in the trichomes of *Arabidopsis*, to learn more about their defense mechanisms, and to apply the knowledge to trichome defense mechanisms in other plant species.

Objectives

The main objective of this study is to observe the induction of PR-1 proteins in trichomes, Columbia wildtype leaves, and Columbia *gl1* mutant leaves when inoculated

with *P. s. pv. phaseolicola*. The *hrp* mutant serves as the control with little or no induction of PR-1. PR-q serves as a marker for the response of plant to pathogen attacks.

Materials and Methods

Growth

The *Arabidopsis* Columbia wildtype and *gl1* mutant seeds will be planted in a tray containing six squares. Seeds will be sowed on soil with the use of paper to spread them over the square pots. The Columbia wildtype was selected because of its high density of trichomes per leaf. The potting soil will contain extended time release fertilizer, be moist, and not compact. A plastic covering will be put over top until the seeds have germinated. Then they will be incubated in a cold room, three to four degrees Celsius for two to four days for stratification. The tray will be placed into a light room in a greenhouse for growth. Around the base of the pots two centimeters of water will be maintained during the germination stage. After this stage the plants will be watered as needed and the plastic covering will be taken off.

Infection

Pseudomonas syringae pv. phaseolicola (1448a) and its *hrp* mutant (TTSS-) will be stored as frozen stock solution at -80 degree Celsius. The bacteria will be grown, prepared and sprayed for inoculations as described in Zipfel et. al. (2004). Fifty to ninety milliliters of bacterial solution will be sprayed onto a flat of seedlings. The treatments that are to be given to both Columbia wildtype and *gl1* mutant seedlings: 1448a, 1448a (TTSS-), and water. The seedlings will be five to six weeks old when inoculated.

Isolation of Trichomes

Isolation Method Number One:

Tubing will be connected to the vacuum and a funnel will be employed upside down to hold the tubing in place. Rubber band will be used around the funnel and tubing to hold them together. A 42.5 millimeter Whatman filter will be used over the tubing to keep the leaf from being sucked down the tube. One leaf will be extracted in the three to five leaf range in the rosette using forceps. The vacuum will create suction to keep the leaf on the filter paper over the hole of the tubing. The scalpel will be dipped in liquid nitrogen to cool the blade. Scalpel will be scraped across the surface of the leaf and the trichomes will be wiped into a micro test tube off of the scalpel. Micro test tube will need to be inspected to ensure that no pavement cells and/or green tissue are present. Liquid nitrogen will be used as often as necessary to keep the leaf and scalpel frozen while extracting the trichomes. The leaf and the material extracted by the scalpel will be checked under a microscope. The slide will be used to verify that the scalpel is extracting just the trichomes. Method will be adjusted until the extraction produces only trichomes. Parafilm will be placed over the micro test tube for storage in –80 degrees Celsius if analysis is not employed immediately.

Isolation Method Number Two:

Two leaves from each treatment for *gl1* mutants and Columbia wildtype will be cut off with a razor blade and stored at -80 degree Celsius. Trichome isolation will be preformed on the three treatments on Columbia wildtype plants as described in Marks et. al. (2008) with one exception. No 60/80 μm glass beads will be used. The trichomes will be stored as described in Marks et. al. (2008) for RNA isolation.

A pilot study will be done to determine what time PR-1 is expressed in leaves and trichomes. Samples will be taken at 24 and 72 hours. The experiment will be replicated three times at the hour that has the highest level of PR-1 induction.

Analysis

The protocol for RNA extraction will be followed according to Rneasy Mini Handbook for Plants and Fungi. The optional DNase Digestion will be used with one change: DNase I incubation mix will incubate for 30 minutes.

Real time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) will be performed as described in Morohashi and Grotewold (2009). The primers to be used are (5'-CTAAGCTCTCAAGATCAAAGGCTTA-3' and 5'-TTAACATTGCAA AGAGTTTCAAGGT-3') for actin, (5'-CTACGCAGAACAACTAAGAGGCAAC-3' and 5'-TTGGCACATCCGAGTCTCACTG-3') for PR-1, or (5'-ATGAAGCTCGTC GGCATGAGTGGG-3' and 5'-TGGATTGCCACTGAGTTGCCTCTG-3') for GL2 (*GLABORUS 2)*.

Statistical Analysis

One sided *t*-test will be calculated to compare the treatment of *P. s. pv. phaseolicola* or its *hrp* mutant normalized with actin to the mock treatment of distilled water. Significance will be based on a p-value less than 0.05.

Results

Figure 1. Trichome Isolation Method

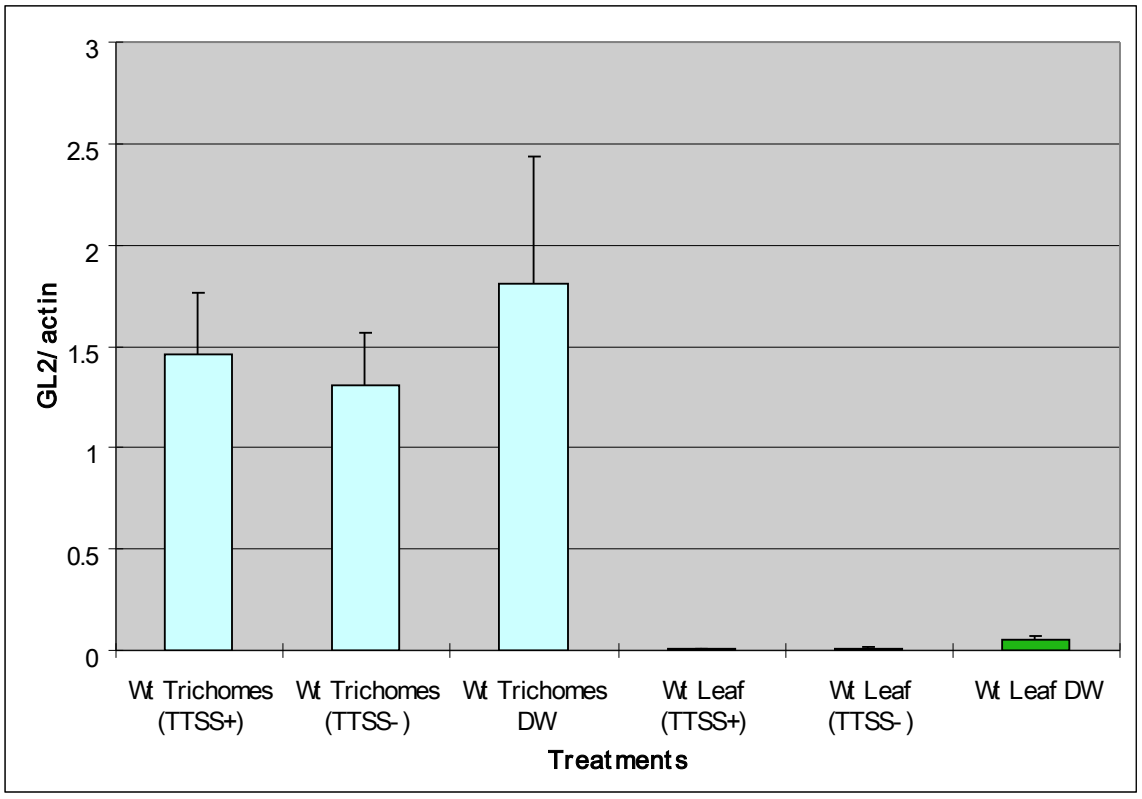
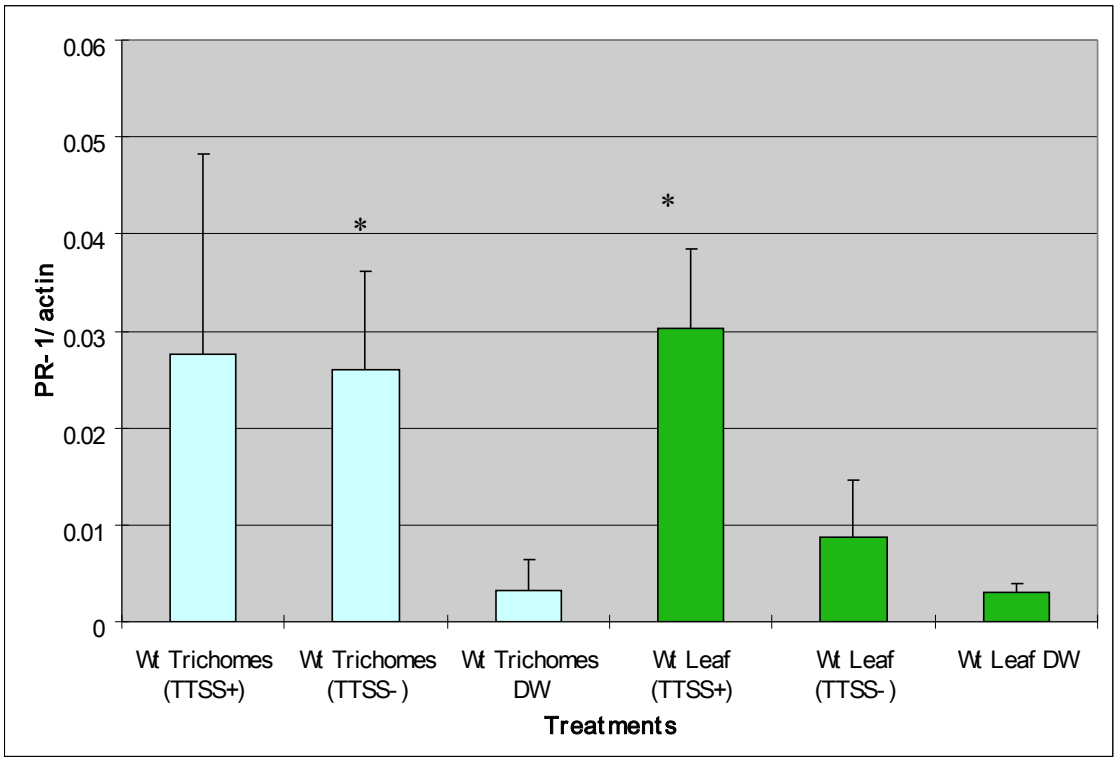


Figure 2. Expression of PR-1/actin in Wildtype Leaves and Trichomes



*, $p<0.05$

Figure 3. Expression of PR-1/actin in Wildtype and *gl1* Leaves

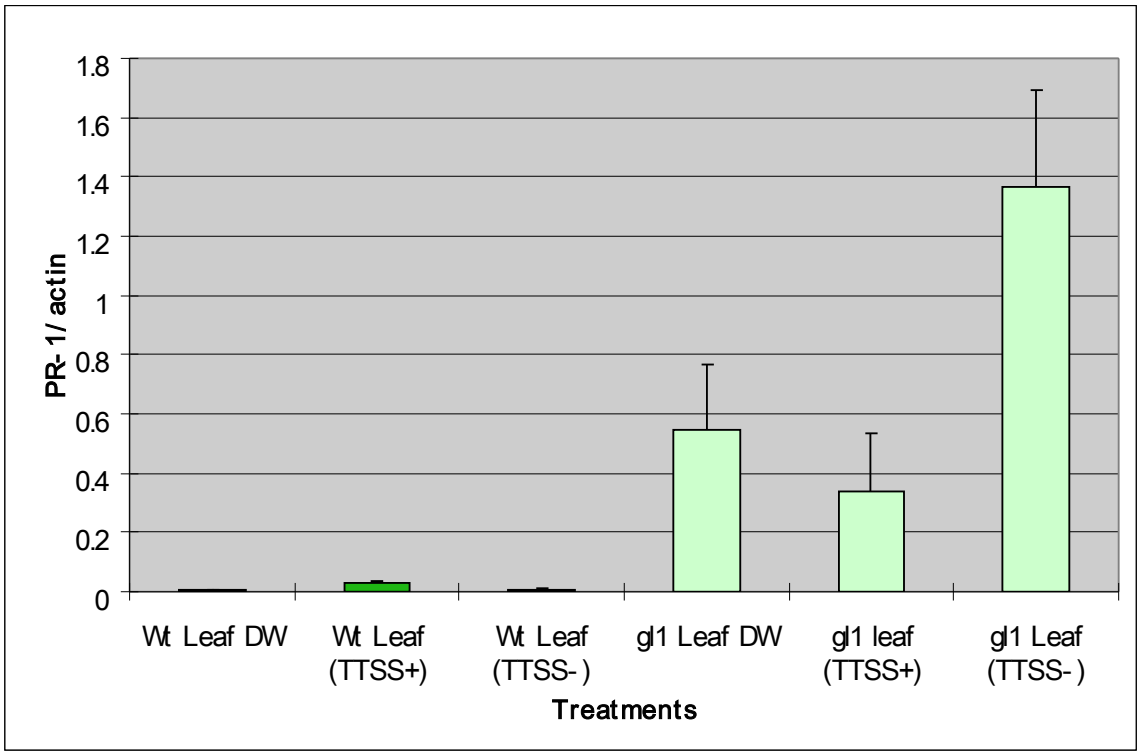
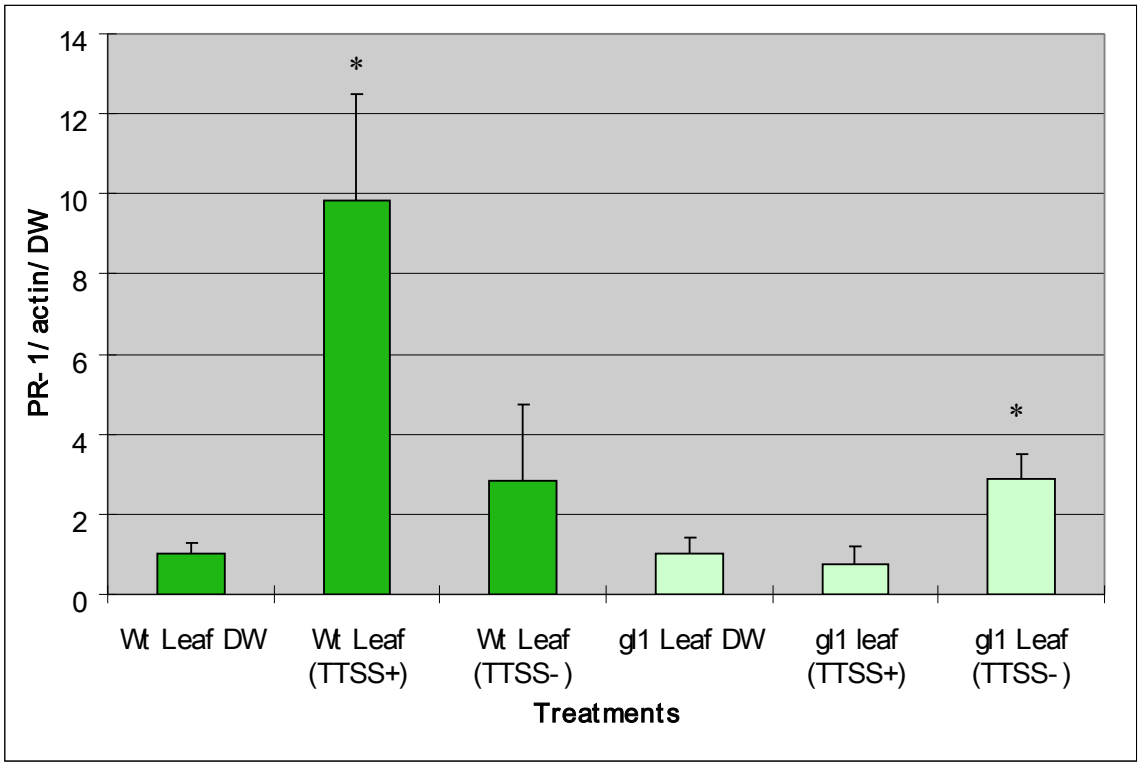


Figure 4. Expression of PR-1/actin/DW in Wildtype and *gl1* Leaves



*, $p<0.05$

Figure 1 demonstrated that the trichome isolation method number two was effective at isolating a large number of trichomes. There the effectiveness of the method was measured by *GL2*, a gene needed for trichome development. High *GL2* levels were present in trichome samples normalized with actin.

Figure 2 shows PR-1 normalized with actin expressed in wildtype trichomes after induced by *P. s. pv. phaseolicola*. *P. s. pv. phaseolicola* and its *hrp* mutant both induce PR-1 response in trichomes. The *hrp* mutant induced PR-1 in wildtype trichomes at significantly high levels (one sided t-test, $p<0.05$). *P. s. pv. phaseolicola* induced PR-1 in wildtype leaves at significantly high levels (one sided t-test, $p<0.05$).

Figure 3 demonstrates that PR-1 normalized with actin is higher in *gl1* mutant leaves than wildtype leaves. *P. s. pv. phaseolicola* (TTSS-) induced high PR-1 levels in *gl1* mutant leaves.

Figure 4 shows levels of PR-1 double normalized with actin and distilled water. *P. s. pv. phaseolicola* induced PR-1 in wildtype leaves at significantly high levels (one sided t-test, $p<0.05$). High levels of PR-1 are induced in *gl1* by (TTSS-) not (TTSS+). *Hrp* mutant (TTSS-) induced significantly high level of PR-1 in *gl1* mutant leaves (one sided t-test, $p<0.05$).

Conclusions and Future Work

Evidence of PR-1 in trichomes strongly suggests that trichomes have a defense mechanism to detect pathogen infection. PR-1 expression in trichomes is independent of TTSS of *P. s. pv. phaseolicola*. Evidence from figure 1A in Ham et. al. (2007) supports the statistically significantly high PR-1 levels found in Columbia wildtype leaves after induced with *P. s. pv. phaseolicola*. This study also gives evidence for loss of the *GL1* genes to

play a role in defense mechanisms against pathogens. Strong induction of PR-1 was observed in *gl1* after infection with the *hrp* mutant. Methods used in this study maybe applied in the future to discover if *Arabidopsis* trichomes provide a chemical barrier to pathogen infection.

Future studies may include using another glabrous mutant, *gl3* (*GLABROUS 3*), to observe if the response with *gl1* mutant is from the lack of trichomes or the *GL1* gene. Another study could be to look at metabolite accumulation in trichomes after infection with *P. s. pv. phaseolicola* and its *hrp* mutant. Results from this study and future studies on *Arabidopsis* trichomes will be applicable to pathogen induced trichome defense mechanisms in other plant species.

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